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Analysis of Mother-to-Infant Transmission of Hepatitis C Virus: Quasispecies Nature and Buoyant Densities of Maternal Virus Populations

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Mother-to-infant transmission of hepatitis C virus (HCV) was analyzed by sequencing of viral RNA and semiquantitative polymerase chain reaction following ultracentrifugation of maternal sera. In two mother-infant pairs, the hypervariable region 1 (HVR1) and carboxyl terminus of envelope 1 (E1) were sequenced. Both viral sequences in the infants were less diverse than those of their mothers. Although the E1 sequences were almost identical in each mother-infant pair, the HVR1 sequences of the infants were related, but not identical, to those of the mothers. Serial examinations of one infant revealed that the HVR1 nucleotide sequence did not change from 10 days to 3 months of age. In six mothers with uninfected infants, all of the dense fractions of sera contained significant amounts of HCV RNA, whereas in six mothers with infected infants, only two of those fractions contained significant amounts of HCV RNA. These results indicate that the strains of HCV detected in the infants were not dominant in the mothers, but were still transmissible to the infants. As dense fractions are known to contain antibody-bound HCV particles, maternal antibodies against HCV may inhibit viral transmission. ***J. Med. Virol. 51:225–230, 1997.***

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INTRODUCTION

Hepatitis C virus (HCV) is known to be transmitted most efficiently by transfusion of HCV-contaminated blood or blood products and by sharing of contaminated needles among intravenous drug users [Alter, 1994]. In general, 20–40% of HCV-infected adult patients or blood donors have a history of transfusion. On the other hand, at least 35% of adult patients infected with HCV have no identifiable source [A-Kader and Balistreri, 1993].

Several authors [Inoue et al., 1991; Thaler et al., 1991; Nagata et al., 1992; Kuroki et al., 1993; Meisel et al., 1995] have reported on mother-to-infant transmission of HCV. The transmission rate seems to be 5–10% if the mothers are infected with HCV alone [Ohto et al., 1994; Ni et al., 1994; Giacchino et al., 1995]. Coinfection with human immunodeficiency virus (HIV) is known to increase the transmission rate of HCV [Zanetti et al., 1995]. Maternal viral load has been reported to correlate with the risk of transmission [Ohto et al., 1994], although the exact mechanism of mother-to-infant transmission of HCV has not yet been clarified.

The N-terminal region of the putative envelope protein 2 (E2) of HCV shows marked variability in nucleotide and deduced amino acid sequences. This region has been designated hypervariable region 1 (HVR1) [Hijikata et al., 1991]. Analyses of the HVR1 have indicated that multiple subpopulations of HCV exist in a chronically infected patient [Tanaka et al., 1992], and these multiple subpopulations are termed quasispecies [Domingo et al., 1985]. The formation of quasispecies is influenced by the host humoral immune response [Kumar et al., 1994; Kudo et al., 1995], and tentative neutralizing antibodies are thought to play an important role in their formation [Kato et al., 1993; Yamaguchi et al., 1994; Yoshioka et al., 1996].

Recently, HCV has been reported to form immune complexes in the sera of chronically infected patients, and ultracentrifugation studies suggest that the densities of the circulating viral particles become higher if

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they are bound to antibodies [Miyamoto et al., 1992; Tsai et al., 1995]. A relationship between the density of the circulating HCV particles and infectivity of the serum also has been reported [Hijikata et al., 1993].

We identified HCV-infected infants in a prospective setting and then analyzed partial HCV sequences in two mother-infant pairs. In addition, we determined the densities of virus in the sera of mothers who did or did not transmit HCV to their offspring.

MATERIALS AND METHODS

Patients

Since 1991, infants born to HCV-infected mothers have been enrolled in a prospective study of mother-to-infant transmission of HCV and evaluated at five centers in the Nagoya district every 3 months. In 1994, 71 infants born to 71 mothers with positive second-generation HCV antibody tests during pregnancy were followed for 6–40 months. None of the infants had a history of transfusion. HCV infection in the infants was diagnosed by detecting serum HCV RNA. Seven infants were found to have been infected. Four of seven infants had persistent HCV RNA in their sera for more than 6 months. The remaining three infants had HCV RNA detected only transiently in their sera. Two pairs of mothers and infants with persistent infection were analyzed for HCV sequences in their sera according to the method described below. In addition, sera from six of seven mothers who transmitted HCV to their offspring were analyzed for the density of the virus and compared with sera from six mothers who did not transmit HCV.

The study was approved by the Ethics Committee of Nagoya University School of Medicine. The care providers of the infants were informed and gave consent to participate in the study.

Anti-HCV Assay

Second-generation anti-HCV antibodies were assayed using an anti-HCV assay kit (Abotto, Tokyo) or Immucheck (Int. Reagents Corp, Kobe) at each center.

Reverse transcription Polymerase Chain Reaction (PCR)

The PCR assay for detecting the 5′ noncoding region of HCV was carried out as described previously [Shibata et al., 1991]. In brief, total RNA was extracted from 100 μl of serum by a modified acid-guanidine-phenol-chloroform method [Chomczynski and Sacchi, 1987] and dissolved in 7 μl of RNase-free water. cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (BRL, Gaithersberg, MD) in a 20 μl reaction mixture. The cDNA was amplified by 40 cycles of thermal reaction (consisting of denaturation for 1 minute at 94°C, annealing for 2 minutes at 55°C, and extension for 3 minutes at 72°C). A 10 μl aliquot of the amplified PCR product was subjected to agarose gel electrophoresis and stained with ethidium bromide. After transferring to a nylon membrane, the DNA was hybridized with an alkaline phosphatase labeled internal oligonucleotide probe corresponding to the target sequence. The hybridized DNA was detected using the insoluble substrate of 5-bromo-4-chloro-3-indolyl-phosphate/4-nitro-blue tetrazolium chloride (BCIP/NBT) (Sigma, Tokyo). The sensitivity of this method for detecting HCV RNA was estimated to be as high as 10^2 copies/ml of serum using limiting dilution of the cloned PCR product. For semiquantitation of HCV RNA in fractionated sera, the result was described as a strong signal, or "++", if the HCV RNA was detected following ethidium bromide staining of the gel, and as a weak signal, or "+", if detected only after Southern hybridization.

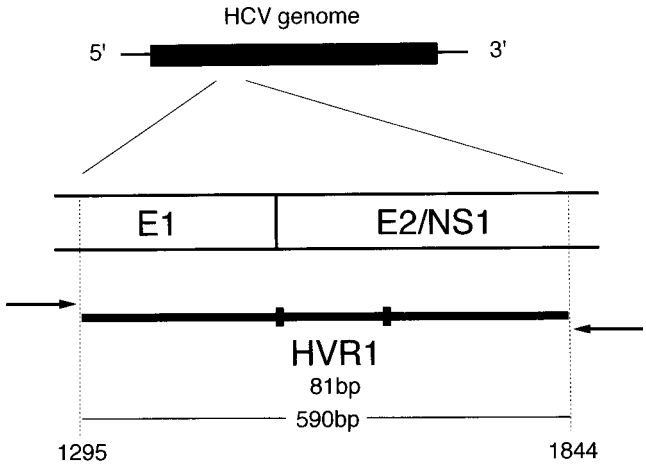

Fig. 1. The analyzed portions of E1 and HVR1 of HCV. They are amplified by the indicated oligonucleotide primer pairs. bp, base pairs; E2/NS1, envelope 2/nonstructural 1 region.

Quantitation of HCV RNA in the Sera

Quantitative detection of HCV RNA [Urdea et al., 1990; Lau et al., 1993] was undertaken using the branched DNA signal amplification assay according to the manufacturer's instructions (Chiron, Emeryville, CA).

Cloning and Sequencing of the PCR Products

Viral sequences were analyzed in the 3′ region of envelope 1 (E1) and the 5′ region of envelope 2/nonstructural 1 region (E2/NS1) (Fig. 1). The target region was amplified once, and the products were cloned following purification by agarose gel-electrophoresis and ligation to the plasmid vector pCR (Stratagene, La Jolla, CA). Recombinant clones were identified by restriction enzyme digestion, and purified plasmids were subjected to DNA sequencing. Thirty cycles of a cycle-sequencing reaction were carried out using Dye-primer (ABI, Tokyo) or Dye-terminator (ABI, Tokyo), both based on the dideoxynucleotide chain termination method [Sanger et al., 1977]. The reaction products were ethanol precipitated and analyzed by a 373A DNA sequencer (ABI, Tokyo). Sequences were analyzed using Genetyx (6.2.0) and Homology (2.2.2) software packages (S.D.C., Tokyo). HCV-BK was selected as a

TABLE I. Characteristics of HCV-Infected Mothers and Semiquantitation of HCV RNA in Fractionated Sera

Case no.	Maternal history of transfusion	Mode of delivery	Breast-feeding	Quantity of HCV RNA[a]	Signal of HCV RNA	
					Top fraction	Bottom fraction
A. Mothers with Infected Infants						
1	+	Vaginal	+	1.1	+	+
2	−	Vaginal	+	1.3	−	+
3	+	C-section	−	<0.5	+	+
4	−	Vaginal	−	<0.5	−	+
5	−	Vaginal	−	2.1	−	++
6	−	Vaginal	−	7.9	+	++
B. Mothers with Uninfected Infants						
7	−	Vaginal	+	<0.5	+	++
8	unknown	Vaginal	+	16.0	+	++
9	−	Vaginal	+	<0.5	−	++
10	−	C-section	+	3.1	+	++
11	+	Vaginal	−	1.5	−	++
12	−	Vaginal	+	2.9	+	++

[a]Assayed by bDNA method (Meq/ml [mega equivalents/ml]).

reference sequence, with base numbers 1295–1481 used as a control for E1, and base numbers 1482–1562 for the HVR1.

Genotyping of HCV RNA

Genotyping was carried out as described previously by Okamoto et al. [1992], and the results were reported according to the method of Simmonds et al. [1993].

Differential Flotation Centrifugation

Differential flotation centrifugation was carried out according to the methods described originally by Havel et al. [1955] as modified by Hijikata et al. [1993]. Twenty microliters of each serum sample were loaded on 1 ml of a sodium chloride solution with a density of 1.063 g/ml, and were centrifuged in a Beckman TLA100.4 rotor at 139500*g* for 22 hours at 14°C. Following centrifugation, 100 μl of the top and bottom fractions were collected, and assayed both by PCR to detect HCV RNA semiquantitatively and by refractometry to determine the respective densities.

Statistical Analysis

Fisher's exact probability test and Student's *t* test were applied for statistical evaluation. A value of $P < .05$ was accepted as statistically significant.

RESULTS

Characteristics of the mothers analyzed are shown in Table I.

Comparison of Partial HCV RNA Sequences of Mothers and Infants

Mother-infant pairs for cases 1 and 3 (Table I) were analyzed. In both infants, HVR1 nucleic acid sequences were less diverse than in their mothers. Homology within the HVR1 in the mother from case 1 was 83–100%, while that in HVR1 in the infant was 99–100%. The mother from case 3 showed 95–100% homology, and her infant showed 99–100% homology.

In both pairs, nucleic acid sequence from the carboxyl terminus of the E1 region showed significant homology between the mother and infant. Sequences from both cases were distinct from other HCV sequences reported in the GenBank, including HCV-BK, which was selected as a reference (Table II).

In contrast, each pair showed marked differences in the HVR1, which is adjacent to the carboxyl terminus of E1, in both nucleotide and deduced amino acid sequences.

Serial Examination of the HVR1 in One Infant

Serial examinations of the HVR1 in the infant from case 3 showed no changes in nucleic and amino acid sequences until the infant was 5 months old, when alterations were observed in two nucleic acids, and in the two deduced amino acids (Fig. 2). His anti-HCV titers had fallen from birth until 5 months of age, when they began to rise (data not shown).

Comparison of the Densities of Circulating Virus in Maternal Sera

Twelve serum samples collected during the perinatal period from 12 mothers were subjected to quantification of total HCV RNA, differential flotation centrifugation, and semiquantitation of HCV RNA in the fractionated sera. Six of the mothers had transmitted the virus to their infants, and the remaining six had not. The characteristics of the mothers are shown in Table I. There was no significant difference in the quantity of total HCV RNA detected between the mothers with infected infants and those with uninfected infants in the sera tested prior to fractionation by ultracentrifugation. Four of six mothers with infected infants had HCV RNA of type 1b, and the remaining two had type 2a. Five of six mothers with uninfected infants had type 1b and one had type 2a.

The densities of the top fractions ranged from 1.07 to 1.08 g/ml, whereas those of the bottom fractions were all greater than 1.15 g/ml. In the bottom fractions, all six serum samples from mothers with uninfected infants showed strong signals for HCV RNA. In contrast,

TABLE II. HCV Sequence Homology Between Mothers and Infants*

Case no.	Comparison with the mother		Comparison with HCV-BK	
	E1	HVR1	E1	HVR1
1	96–99 (95–100)	74–80 (52–60)	91–94 (92–97)	65–66 (56–59)
3	98–100 (98–100)	84–86 (70–74)	91–93 (95)	64 (48)

*Data presented as the range of sequence homology in nucleotides (in deduced amino acids, in parentheses). HCV-BK was used as the reference for HCV genotype 1b. HVR1, hypervariable region 1.

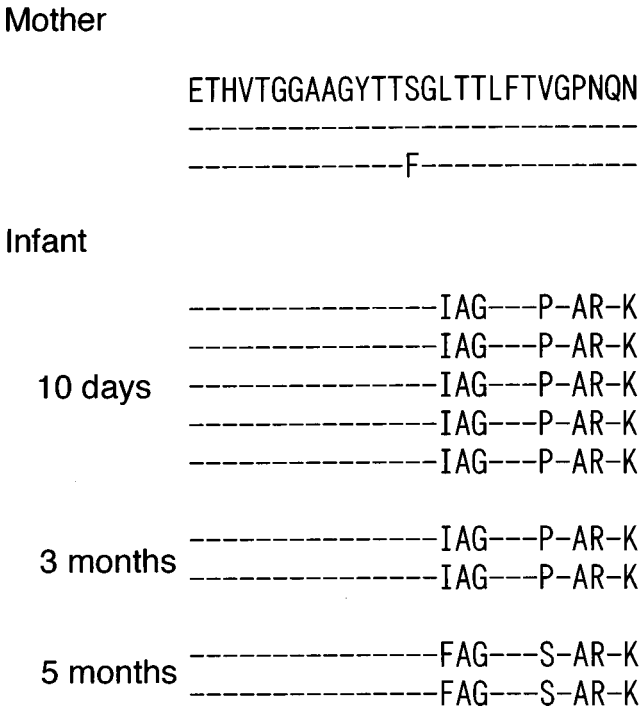

Fig. 2. Serial examination of HVR1 in an HCV-infected infant from case 3. Deduced amino acid sequences from the major clones of the mother are indicated by single-letter codes. Letters in infant's sequences denote amino acids different from those in the mother. The nucleotide or amino acid sequence of the infant is composed of a single strain until 3 months of age, and shows no change.

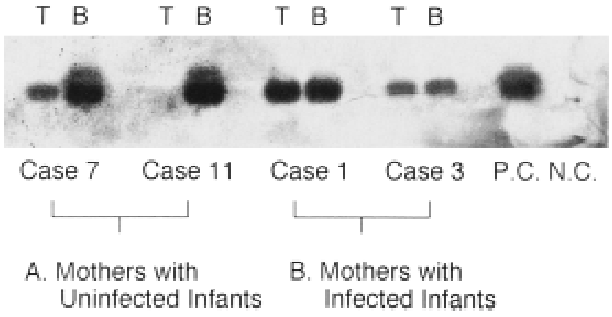

Fig. 3. Signals of HCV RNA from each fraction of maternal sera. Case number, see Table I; T, top fraction; B, bottom fraction; P.C., positive control; N.C., negative control.

only two of the six serum samples from mothers with infected infants revealed strong signals for HCV in the bottom fractions ($P < .05$). Signals for the PCR products are shown in Figure 3.

DISCUSSION

This study provides evidence for the existence of an infective subpopulation among quasispecies of maternal HCV, and for the possible influence of antibodies against HCV on perinatal transmission.

First, the marked difference in the HVR1 sequences within each mother-infant pair and the identification of less diverse populations in infants than in mothers suggest that a minor but infective subpopulation of virus in the mothers was transmitted. Weiner et al. [1993] have described similar observations in mother-to-infant transmission of HCV. In the case of HIV, which also forms quasispecies within an infected individual, differences in the V3 loop sequences from three mother-infant pairs also have been reported [Wolinsky et al., 1992].

Second, no nucleic or amino acid changes were observed in the case 3 infant from 10 days to 3 months of age, which argues against the possibility that the high mutation rate of HCV accounts for the differences in HVR1 between the mother and infant. Our results also emphasize the role of the humoral immune response in the formation of HCV quasispecies, and they are consistent with an earlier observation regarding the HVR1 in an agammaglobulinemic patient, whose envelope glycoprotein remained unchanged for 2.5 years [Kumar et al., 1994].

Third, the mothers with uninfected infants more frequently had high amounts of HCV RNA in the high-density fractions of their sera than the mothers with infected infants. Densities of the virus are believed to increase in reaction to elements of the humoral immune response, some of which may be caused by neutralizing antibodies, and by the production of naked nucleocapsids [Kanto et al., 1994, 1995a,b]. Hijikata et al. [1993, 1995] have reported that low-density virus is more highly infective to chimpanzees than high-density virus, and that the latter is associated with the HCV-specific immunoglobulin. Our results support these findings. However, we could not recover from the top fractions of maternal sera, the infectious subpopulation found in the infants (data not shown).

Although our system was not sufficiently sensitive to detect these infectious subpopulations in fractionated sera, it is possible that the maternal immune response resulted in inhibition of mother-to-infant transmission by reducing the amount of infectious viral particles in

the circulation. This is consistent with the observation that the dominant populations of maternal virus were not transmitted.

Two mothers with infected infants showed high levels of HCV RNA in the bottom fractions of their sera. This suggests not only that the density of circulating virus is a key factor in transmission, but that other factors probably play a role as well.

In Table I, two of six mothers of infected infants and five of six mothers of uninfected infants have breast-fed. In our recent series, however, there was no significant difference in the rates of breast-fed infants between those who are infected and those who are not (4 [50%] of 8 infected infants were breast-fed, and 56 [73%] of 77 uninfected infants were breast-fed $P = .18$, Fisher's exact test, unpublished data). Breast-feeding did not seem to affect mother-to-infant transmission of HCV, although breast milk was supposed to contain antiviral components which interfere with mother-to-infant transmission of HIV [Van de Perre et al., 1993].

Routes of mother-to-infant transmission of HCV are not yet well defined. Our case 3 infant was abdominally delivered and was not breast-fed, which supports the possibility of transplacental HCV transmission. However, further studies are required to define the timing of transmission [Kuhn and Stein, 1995]. Delivery is always associated with mechanical dissociation of the placenta and the uterus, which may cause a mixing of blood between the mother and infant.

In conclusion, virologic analysis of mother-to-infant transmission of HCV suggests a significant role for the maternal humoral immune response in preventing transmission of the virus.

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